# STUDIES ON A BACTERICIDAL AGENT EXTRACTED FROM A SOIL BACILLUS

## I. Preparation of the Agent. Its Activity in Vitro

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Microorganisms perform a vast number of biochemical reactions, many of which are not known to occur in the animal and plant kingdoms (1). On the basis of present knowledge it is conceivable that one may find in nature microbial species endowed with catalysts capable of activating almost any type of biochemical reaction. During the past few years, this point of view has found its application in the isolation of soil microorganisms which selectively attack certain substances of interest to the biochemist (2) and to the immunologist (3–8). It may be recalled in particular that soluble polysaccharides, extracted from several bacterial pathogens, have been found to be decomposed by certain microbial species, although the same substances are resistant to the action of all known enzymes of animal and plant origin.

It appeared possible that there also exist in nature microorganisms capable of attacking not only isolated soluble components of other bacterial cells, but also the intact living cells themselves. Actually we have isolated from soil a spore-bearing bacillus which attacks and lyzes the living cells of several species of Gram-positive microorganisms. The present paper describes the isolation of this new soil bacillus, and the preparation, properties, and activity of the soluble agent by means of which it attacks and lyzes the living cells of the susceptible, Gram-positive species.

### EXPERIMENTAL

*Isolation of a Sporulating Bacillus Capable of Lyzing the Living Cells of Gram-Positive Microorganisms.*—The method employed for the discovery of microorganisms capable of attacking certain definite organic compounds has already been described (2, 3). It is based on the assumption that all organic matter added to the soil eventually undergoes decomposition through the agency of microorganisms. In the present case, it was hoped

that the addition to soil of living cultures of Gram-positive cocci would result in the development of a selective soil flora capable of attacking the living cells of these bacterial species.

Soil samples of neutral reaction, obtained from a number of different sources, were pooled, and mixed with alkaline phosphate, ammonium sulfate, and an excess of calcium carbonate. The mixed sample was kept at about 70 per cent of its moisture-holding capacity and incubated at 30°C. for a few weeks in order to bring about the decomposition of most of the organic matter originally present.

Staphylococci, pneumococci (R variants), and group A hemolytic streptococci (glossy variants) were grown in beef infusion peptone broth and centrifuged; the living cells, resuspended in small volumes of distilled water, were added to the soil sample. After two years, during which this process was repeated at irregular intervals of time, a small amount of the soil preparation (2 gm.) was added to 10 cc. of mineral medium (M/20 $K_2HPO_4$, M/30 $NaH_2PO_4$, M/100 $(NH_4)_2SO_4$, tap water) to which had been added the living cells recovered from 150 cc. of staphylococcus culture. This suspension, containing soil and staphylococci, was incubated in shallow layers at 30°C. After 48 hours incubation, stained films of the bacterial suspension revealed advanced lysis of the staphylococci and the presence of a mixed bacterial flora. A small amount of the same suspension was now transferred to a similar medium and incubated under the same conditions. Again, lysis of the staphylococci was observed within 48 hours. A few more transfers were sufficient to eliminate most of the microbial species originally present in the soil used as inoculum. By plating on peptone agar, it was finally possible to isolate in pure culture a Gram-positive bacillus capable of lyzing living staphylococci resuspended in the mineral medium described above.

*Description of the Organism.*—The organism is a motile spore-bearing bacillus measuring in average $4 \times 0.5\ \mu$. The spores are terminal, oval; they resist heating at 80°C. but are killed at boiling temperature.

Very young cells of the bacillus retain the Gram stain, but they soon lose this characteristic and appear then as Gram-negative rods. This change in staining reaction is probably associated with the fact that cultures undergo autolysis very rapidly (24 to 48 hours), giving rise to shadow cells with intensely stained granules, Gram-negative detritus, and a mass of oval spores.

The organism grows readily and abundantly on peptone media, especially when incubation is carried out in shallow layer at 37°C. No gas is formed in sugar peptone media, and the culture does not become acid (the following substrates were tested; arabinose, dextrose, dulcite, inulin, lactose, mannose, mannite, rhamnose, saccharose, salicin, sorbitol, xylose). Litmus milk becomes alkaline. The fact that indicators do not reveal the production of acid does not necessarily mean, however, that the substrates are not fermented by the culture. It has been observed that the growth of the bacillus in peptone solution renders the medium very alkaline (pH 9.0) and it is possible that the production of alkaline substances may mask the production of organic acids from the sugars added to the peptone.

The organism liquefies gelatin and gives a diffuse zone of hemolysis on blood agar plates. It gives a negative V.P. test and forms $H_2S$ on lead acetate agar medium. Catalase production is very abundant.

*Preparation and Properties of a Soluble Bacterial Extract Which Attacks the Living Cells of Gram-Positive Microorganisms.*—As stated above, the spore-bearing bacillus just described is capable of lyzing suspensions of living staphylococci. The lysis is caused by an agent which is found in solution in autolysates of cultures of the soil bacillus. When very young cultures (12 to 18 hours) of the bacillus are used, the active agent is found in the bacterial bodies; it can then be obtained in solution by collecting the bacterial cells, and allowing them to autolyze in an aqueous medium. As the culture becomes older, however, more and more of the active material is found in solution in the culture medium and, after 72 hours (when practically all cells have autolyzed) only a small percentage of the activity is found associated with the cellular material.

The active principle is quantitatively precipitated at pH 4.5; the precipitate, redissolved at neutral reaction, exhibits the activity of the original solution.

On the basis of these observations, the following method has been employed for the preparation and concentration of the active extract used in the experiments described in this and the following paper.

The organism is grown in a medium consisting of 1 per cent acid hydrolysate of casein in tap water, at pH 7.0. The culture is incubated in shallow layer (3 cm.) for 3 to 4 days at 37°C. At the end of the incubation period, stained films of the culture show the presence of Gram-negative shadow forms, and of large numbers of spores, and the reaction of the medium has become alkaline (pH 9.0). The culture is then centrifuged for 1 hour at 3,500 R.P.M. The clear supernatant fluid is separated, and acidified to pH 4.5 (this requires about 4 cc. of concentrated HCl per liter of autolysate). A faint precipitate forms; it is separated by centrifugation or filtration and redissolved at neutral reaction.

Work is now in progress toward further purification of the active material; a complete description of its properties and composition is therefore reserved for the future, when pure preparations are available. It may be stated, however, that the preparations obtained by the technique just described give all the protein tests and contain 14.5 per cent nitrogen. The active principle does not dialyze through collodion membranes; it retains its activity after 10 minutes heating at 90°C. at pH 2.0 and pH 9.0. It is also resistant to the action of pepsin, of crystalline trypsin and chymotrypsin, and of crude trypsin. It must be pointed out, however, that although treatment with heat or with proteolytic enzymes does not inactivate the bactericidal agent, it renders it insoluble in neutral buffer solutions.

Filtration through Berkefeld candles (V) gives preparations which are capable of lyzing the susceptible bacterial species although much activity is lost during the process. Filtration through Chamberland filters or asbestos pads has always resulted in complete loss of activity.

*The Effect of the Soluble Bacterial Extract upon the Living Cells of Several Microbial Species.*—The effect of the bacterial extract upon different microbial species has been studied under a variety of conditions which will be described in this and other publications. It is permissible to state at the present time, however, that the bacterial extract has been found to exert a marked bactericidal effect upon all the Gram-positive species thus far tried, whereas all the Gram-negative bacilli have remained unaffected. Gram-negative cocci and acid-fast bacilli have not yet been tried.

The following microbial species have been studied:

*A. Gram-positive species* (all susceptible to the bactericidal effect of the extract). *Diplococcus pneumoniae:* Five strains of virulent encapsulated pneumococci (Types I, II, III, V, and VIII). Three strains of avirulent rough pneumococci (derived from Types I, II, III). *Streptococcus hemolyticus:* Eight matt and glossy strains of group A (types 1, 3, 6, 14, 20). Three virulent strains of group C. Three strains of group D (cheese streptococci). *Streptococcus viridans:* Two strains. Indifferent (gamma) streptococci: Two strains. *Staphylococcus aureus:* One rabbit virulent strain, one mouse virulent strain. Unidentified culture of spore-bearing Gram-positive bacillus. *Saccharomyces cerevisiae.*

*B. Gram-negative species* (not susceptible to the bactericidal effect of the extract). *Escherichia coli. Eberthella typhi. Salmonella paratyphi. Klebsiella pneumoniae* type B. *Hemophilus influenzae.* Unidentified culture of spore-bearing Gram-negative bacillus.

The activity of the bacterial extract upon the cells of the different microbial species, as measured by lysis and loss of viability, was studied in the following experiments.

The test cultures were grown for 8 hours in meat infusion peptone broth; the cells were separated by centrifugation and resuspended in phosphate buffer (pH 7.6) to give suspensions containing about $10^9$ cells per cc. The suspensions, distributed in 1 cc. amounts into test tubes, were treated with different amounts of bacterial extract (preparation NS7) and made up to a final volume of 2 cc. with phosphate buffer (see Table I).

After 3 hours incubation at 37°C. the different preparations were observed for the occurrence of lysis (turbidity readings, confirmed by microscopic examinations), and streaked on blood agar plates to determine the effect of the bacterial extract on the viability of the cells (Table I).

TABLE I

*Effect of a Soluble Bacterial Extract on Different Microbial Species*

| Test organisms | | Amount of extract used (mg. per $10^9$ cells) | | | | |
|---|---|---|---|---|---|---|
| | | 5 | 1 | 0.1 | 0.01 | 0 |
| *Diplococcus pneumoniae* (8 strains) | Lysis* | C | C | C | P | N |
| | Viability† | − | − | − | − | ++++ |
| | Reductase‡ | NR | NR | NR | NR | CR |
| *Streptococcus hemolyticus* group A (7 strains) | Lysis | P or N | N | N | N | N |
| | Viability | − | − | − | − | ++++ |
| | Reductase | NR | NR | NR | NR | CR |
| *Streptococcus hemolyticus* group C (3 strains) | Lysis | P or N | N | N | N | N |
| | Viability | − | − | − | − | ++++ |
| | Reductase | NR | NR | NR | NR | CR |
| *Streptococcus hemolyticus* group D (3 strains) | Lysis | N | N | N | N | N |
| | Viability | − | + | ++++ | ++++ | ++++ |
| | Reductase | NR | PR | CR | CR | CR |
| *Streptococcus viridans* (2 strains) | Lysis | P or N | N | N | N | N |
| | Viability | − | − | − | − | ++++ |
| Indifferent streptococcus (2 strains) | Lysis | N | N | N | N | N |
| | Viability | − | − | − | − | ++++ |
| *Staphylococcus aureus* (2 strains) | Lysis | C | C | P | N | N |
| | Viability | − | − | − | ++++ | ++++ |
| | Reductase | NR | NR | NR | CR | CR |
| Gram-positive spore-bearing bacillus | Lysis | X | C | X | X | N |
| *Escherichia coli* | Lysis | N | N | N | N | N |
| | Viability | ++++ | ++++ | ++++ | ++++ | ++++ |
| | Reductase | CR | CR | CR | CR | CR |
| *Klebsiella pneumoniae* group B | Lysis | N | N | N | N | N |
| | Viability | ++++ | ++++ | ++++ | ++++ | ++++ |
| | Reductase | CR | CR | CR | CR | CR |
| *Hemophilus influenzae* | Lysis | N | N | N | N | N |
| | Viability | ++++ | ++++ | ++++ | ++++ | ++++ |
| Gram-negative sporulating bacillus | Lysis | X | N | X | X | N |
| | Viability | X | ++++ | X | X | ++++ |
| *Saccharomyces cerevisiae* | Lysis | N | N | N | N | N |
| | Viability | − | ++++ | ++++ | ++++ | ++++ |
| | Reductase | NR | CR | CR | CR | CR |

* C = complete lysis. P = partial lysis. N = no lysis.

† − = no growth on blood agar. + = much reduced growth on blood agar. ++++ = abundant growth on blood agar.

‡ CR = complete reduction of the methylene blue. PR = partial reduction of the methylene blue. NR = no reduction of the methylene blue. X = not done.

The results presented in Table I indicate that the soluble bacterial extract exerts a bactericidal effect on all the Gram-positive microorganisms so far tested. Unequivocal evidence of lysis was recognized only in the case of pneumococci, staphylococci, and a Gram-positive spore-bearing rod. The soluble extract exerted no bactericidal effect, nor did it cause the lysis of any of the Gram-negative species.

The bactericidal effect of the extract was concluded from the inability of the treated susceptible cells to grow on subsequent transfer to blood agar plates. In other tests, these results were confirmed by inoculating the cells into liquid media, where they failed to multiply, or by injecting the virulent species into susceptible animals, which they failed to kill.

It is worth emphasizing the comparative effects of different amounts of extract in causing the death or the lysis of the various microorganisms. In the case of pneumococci, staphylococci, and the Gram-positive spore-bearing rods, death of the cell is accompanied by lysis. On the contrary, streptococci do not lyze although they are susceptible to the bactericidal effect of the extract; in fact, no lysis of the streptococci is observed even when they are treated with 100 times the minimal amount of extract required to kill the cells. It is also worth noting that staphylococci, although much more resistant than group A hemolytic streptococci to the killing effect of the extract, undergo lysis very readily.

It appears therefore that no parallelism exists between lytic effect and bactericidal effect; in fact, it will be shown later in this article that lysis is only a secondary process, caused by the action of the autolytic enzymes of the cells, and follows some other primary injury inflicted by the bactericidal agent.

Several attempts have been made to compare the effect of the bactericidal agent upon pneumococci and upon group A hemolytic streptococci in different culture phases (rough and smooth, glossy and matt variants). This was studied by determining the minimal amount of extract required to kill the same number of cells of different species in a given time, or by measuring the length of time required by a same amount of extract to kill the same number of cells. Under these conditions no difference could be found between R and S pneumococci, or between glossy, matt avirulent, and matt virulent streptococci, irrespective of type derivation. Furthermore pneumococci, group A and group C hemolytic streptococci, green and indifferent streptococci, were all found to be equally susceptible to the bactericidal agent.

*The Inhibitory Effect of the Bactericidal Agent on the Glucose Dehydrogenase of Microorganisms.*—The results presented in Table I indicate that the

minimal effective dose of the bactericidal agent is not the same for all the microbial species. Yeast and cheese streptococci require the largest amount (1 to 5 mg. per $10^9$ cells), next come the staphylococci (0.1 mg. per $10^9$ cells); the pneumococci, hemolytic streptococci of group A and C, green and indifferent streptococci, are all killed by very small amounts of extract (0.01 mg.). In a general way, it can be said that the resistance of the various microbial species to the bactericidal agent varies in the order of their metabolic activity, as measured by their ability to reduce methylene blue in the presence of glucose: yeast and cheese streptococci (group D) metabolize more actively than staphylococci, which in their turn are more active than pneumococci and hemolytic streptococci of group A. This parallelism suggested that the primary toxic effect of the bactericidal agent might be directed against the dehydrogenase system of the microbial cell; some preliminary experiments were instituted to test this point.

Microbial suspensions containing approximately $10^9$ cells per cc. were used. They were distributed in 3 cc. amounts into test tubes and were treated with different amounts of the bactericidal agent (Table I). The mixtures were incubated for 3 hours at 37°C. Methylene blue (1 cc. of 0.002 M solution) and glucose (1 cc. of 10 per cent solution) were then added, and the mixtures, sealed with vaseline, were incubated at 37°C. The rate of reduction of the dye was observed. Although the data concerning the experimental procedure, and the results obtained, will be presented *in extenso* in another publication, a general summary of the final results is incorporated in Table I.

It is clear that, in the case of all the Gram-positive organisms, incubation of the microbial cells with sufficient amounts of the bactericidal extract results in an inhibition of methylene blue reduction. The glucose dehydrogenase of the Gram-negative bacilli, on the contrary, is not affected by the same treatment.

In all cases, the minimal amount of bactericidal extract required to kill the microbial cells is also sufficient to inhibit their reducing action. In other words, the loss of viability appears to be quantitatively related to the inactivation of the glucose dehydrogenase of the cell.

*Inhibition of Growth by the Bactericidal Agent.*—The lytic effect of the soluble agent upon pneumococci and staphylococci, and its bactericidal effect upon Gram-positive organisms in general, can be observed not only when the susceptible cells are resuspended in buffer solution, but also in the presence of meat infusion, peptone, serum, and ascitic fluid. It was to be expected, therefore, that the extract would inhibit the growth of the susceptible species in culture media.

Inhibition of growth has in fact been observed in the case of all the Gram-positive organisms mentioned in Table I. A single example will illustrate

the activity of the extract in this respect. Test tubes containing 5 cc. of meat infusion peptone broth were inoculated with 0.03 cc. of a pneumococcus culture (D39R), and treated with different amounts of bactericidal agent (preparation NS7). Full growth had developed within 12 hours in the untreated control tubes whereas it took 24 hours for growth to appear in the tube which had received 0.000,01 mg. of extract. No growth developed in the tubes which received 0.000,1 mg. (or more) of the same preparation. On the contrary, no inhibition or retardation of growth was observed with any of the Gram-negative bacilli; for instance *Escherichia coli, Klebsiella pneumoniae, Eberthella typhi,* grow normally in the presence of large amounts of extract, even when an inoculum as small as $10^{-7}$ cc. is used.

## DISCUSSION

The bactericidal agent described in the present paper is associated with a protein fraction which precipitates out of solution at pH 4.5. Heating at 90°C., or digestion with proteolytic enzymes, renders the active fraction insoluble, but does not in any way affect its lytic or bactericidal power, as can be shown by adding the suspension of insoluble material to the susceptible bacterial species. It is possible therefore, that the active substance itself is not a protein, but that the protein with which it is associated determines its solubility properties.

Although the agent exerts a bactericidal effect on the cells of all the Gram-positive species so far tested, its lytic effect has been observed only against pneumococci, staphylococci, and an unrelated Gram-positive spore-bearing bacillus. It is likely, therefore, that the death of the cell does not result from a lytic action of the extract, but that on the contrary, lysis is only a secondary process. The bacteriological literature offers several examples of "secondary lysis" following treatment with various antiseptics (heavy metals, formaldehyde, iodine, bile salts, toluol, acetone, etc.) (9, 10). In fact, the "bile solubility" of pneumococci is a good example of this phenomenon. Bile salts, unsaturated fatty acids, do not by themselves lyze the pneumococci; they inflict upon the cell an injury which destroys some essential metabolic function, without at the same time destroying the autolytic enzymes; the autolytic system, held in abeyance in the normal living cell, then begins to function, and autolysis follows (11, 12).

It is worthy of notice that group A hemolytic streptococci are known to be very resistant to normal autolysis; staphylococci autolyze more rapidly and pneumococci most rapidly of all. It has now been found that the

bactericidal agent considered in the present paper "lyzes" pneumococci most readily, staphylococci somewhat more slowly, and streptococci little or not at all, even though group A hemolytic streptococci are as susceptible as pneumococci to the bactericidal effect of the agent. Furthermore, it has been found that the bactericidal agent does not lyze the cells of pneumococci or staphylococci in which the autolytic enzymes have been destroyed by heating, or by treatment with formaldehyde. All these facts, when taken together, suggest that the bactericidal agent exerts a toxic action upon the living cells of the susceptible species and that lysis, when it occurs, is only a secondary process, caused by the cells' own autolytic enzymes.

How then does the bactericidal agent exert its toxic effect? It has been found that the minimal amount of extract which causes the death of the cell also destroys its ability to reduce methylene blue in the presence of glucose. This is not, however, sufficient evidence to establish that the inhibition of the glucose dehydrogenase is the cause of cell death. It remains possible that both effects are the common result of some other primary injury, as yet unrecognized. In the analysis of this question, it may be important to keep in mind that the minimal amount of extract required to kill a given number of cells is the same whether the test is carried out with pneumococci, or hemolytic streptococci of group A and C, or green and indifferent streptococci, irrespective of type derivation and culture phase (rough and smooth, matt and glossy variants). This observation suggests that the effect of the bactericidal agent is directed against a cellular structure or function which is common to all these bacterial species.

Furthermore, it is worth emphasizing again that the extract appears to be effective only against Gram-positive microorganisms. No lytic or bactericidal effect, no inhibition of glucose dehydrogenase, no retardation of growth could be observed with any of the Gram-negative bacilli so far tested (Gram-negative cocci and acid-fast bacilli have not yet been tested). Many examples are already known of differential toxic action of antiseptics upon Gram-positive and Gram-negative species (13–15). In fact, the Gram stain appears to divide the microbial world into two groups which differ widely, not only in several of their physiologic properties, but also in the chemical structure of the cell (16). Professor Christian Gram, who introduced in bacteriology the staining technique which bears his name, died recently (17). It may be proper at this time to suggest that the Gram reaction, which has proved of such great importance in the identification and classification of microbial species, may in the future serve as a guide in the study of fundamental problems of bacterial physiology and cytology.

SUMMARY

A Gram-positive, spore-bearing, aerobic bacillus, capable of lyzing the living cells of many Gram-positive microbial species, has been isolated from soil.

Cultures of this soil bacillus in peptone media release during autolysis a soluble agent which exerts a bactericidal effect on all the Gram-positive microorganisms so far tested, and inactivates their glucose dehydrogenases. It also inhibits the growth of the susceptible species in culture media.

Several of the Gram-positive species undergo lysis when incubated with the bactericidal agent. It appears however, that lysis is only a secondary process, due to the autolytic enzymes of the susceptible cells, and that it follows upon some other primary injury caused by the active agent.

The bactericidal agent is ineffective against all the Gram-negative bacilli so far tested.

*Addendum.*—The bactericidal agent described in the present paper has now been obtained in a form free of protein; the new purified preparations are about 50 to 100 times more active, both *in vitro* and *in vivo*, than the ones used in the experiments which have just been recorded.

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